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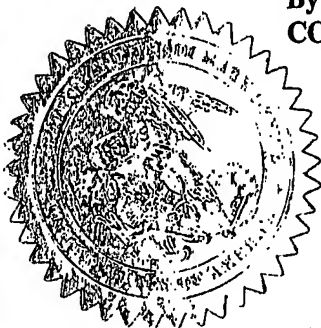
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# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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**USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT**

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PROCESS FOR DESIGNING INHIBITORS  
OF THE LIFE CYCLE OF INFLUENZA A VIRUS  
TARGETED TO THE VIRAL NON-STRUCTURAL PROTEIN 1

The Multiple Functions Of The NS1 Protein Of Influenza A Virus (NS1A Protein) In The  
Intracellular War Between The Virus And Human Cells

The intracellular war between influenza A virus and human cells is comprised of several battles, and the viral NS1A protein plays a major role in many of these battles. The first battle: infection of human cells by influenza A virus activates the interferon (IFN)-independent transcription of antiviral genes mediated by activated interferon regulatory factor-3 (IRF-3) transcription factor. The NS1A protein counters this cellular antiviral response at the post-transcriptional level by binding cellular proteins required for the 3' end processing of cellular pre-mRNAs. Mature antiviral mRNAs are not made unless the virus encodes a NS1A protein with a mutated effector domain binding site for one of these cellular factors (CPSF). This mutation also affects a second cellular antiviral response; IFN-beta mRNA is produced earlier and in larger amounts, because post-transcriptional processing of cellular pre-mRNAs is not inhibited throughout infection. In addition, the NS1A protein is localized in the cytoplasm, indicating that CPSF binding mediates nuclear import/retention of the NS1A protein. Another sequence in the NS1A effector domain, the nuclear export signal (NES, amino acids 137-146) plays an important role, because a recombinant virus encoding a NS1A protein with a mutated NES is substantially attenuated. The N-terminal RNA-binding domain, which binds double-stranded RNA (dsRNA) with low affinity, plays a different role, as demonstrated using recombinant viruses that encode NS1A proteins whose only defect is in RNA-binding: PKR, expressed constitutively in the absence of IFN, is activated, eIF2-

alpha is phosphorylated, and viral protein synthesis is inhibited. After its activation, PKR is degraded.

# The RNA Binding Domain Of The NS1 Protein Of Influenza A Virus: Identification Of One Of Its Functions During Virus Infection

The NS1 protein of influenza A virus (NS1A protein) plays a major role in the intracellular war between the virus and human cells. Functions of the effector domain of the NS1A protein, which comprises the C-terminal two-thirds of the protein, have been elucidated. In contrast, the function of the N-terminal RNA-binding domain of the NS1A protein, which binds dsRNA with low affinity, has not been established. For example, it does not sequester dsRNA to prevent the activation of either IRF-3 or NF-kappaB. Here we determine whether dsRNA binding by this domain prevents activation of PKR during infection. Activated PKR phosphorylates the translation initiation factor eIF2 $\alpha$ , resulting in the inhibition of protein synthesis and virus replication. Substantial amounts of PKR, which can be activated by dsRNA or by specific proteins, are expressed constitutively in the absence of interferon. Previous results from others indicated that the amino acids of the NS1A protein that participate in the inhibition of PKR do not include those that are required for RNA binding. We generated recombinant A/Udorn/72 viruses that encode NS1A proteins whose only defect is in RNA binding. Because the R at position 38 (R38) and K41 are the only amino acids that are required solely for RNA binding, we substituted A for either one or both of these amino acids. The three mutant viruses are highly attenuated: the R38 and K41 mutant viruses form pin-point plaques, and the double mutant (R38/K41) does not form visible plaques. During high multiplicity infection of A549 cells with any of these mutant viruses, PKR is activated, eIF2 $\alpha$  is phosphorylated, and viral protein synthesis is inhibited. Surprisingly, after its activation, PKR is degraded. The R38/K41 double mutant is most effective in inducing PKR

activation. We conclude that one of the intracellular functions of the RNA-binding domain of the NS1A protein is to prevent the activation of PKR by binding dsRNA.

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